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(54) Title: METHOD AND DIAGNOSTIC KIT FOR DETERMINATION OF BACTERIA

(57) Abstract

Biotinylated species or genus specific bacteriophage and an aqueous suspension of solid phase avidinylated particles labeled with an indicator enzyme such as horseradish peroxidase are contacted with a test sample and complex with any organisms of the species or genus specific bacteria in the sample. The complex is separated, preferably by filtration, from smaller size particles and a color developing reagent is applied to the filtration medium. Development of color is indication bacteria of the specific type are present in the sample tested.

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Title: METHOD AND DIAGNOSTIC KIT FOR DETERMINATION OF BACTERIA

Background of the Invention

Field of the Invention

This invention relates to microbiology, and more particularly, to a method and kit using bacteriophage for the determination of the presence of organisms of a specific bacteria type in a sample.

Background of the Invention

Foodborne disease has been estimated to cause at least six million illnesses and more than a billion dollars in economic losses each year in the United States. There are an estimated 800,000 to 4,000,000 *Salmonella* infections in the United States each year, and at least 500 deaths. At least two million cases of *Campylobacter* infections are estimated to occur each year in the United States, with estimates of deaths associated with *Campylobacter* infections ranging from 200-800 annually. The increase in the number of *Salmonella* outbreaks has been unrelenting. From the period 1973-1975 to 1985-1987, there was a 75% increase in the proportion of bacterial outbreaks caused by *Salmonella*, and outbreaks of *Clostridium botulinum* intoxication, *Listeria* infections, and *Salmonella* infections accounting for 75% of the known deaths. Testimony of the Director, Div. of Bacterial and Micrological Diseases, National Center for Infectious Diseases, Centers for Disease Control, Public Health Service, Dept. of Health and Human Services, before the Committee of Human and Labor Resources of the United States Senate on June 28, 1991.

Recent epidemiological evidence suggests that the number of cases of Campylobacteriosis in the United States exceeds the number of cases of *Salmonellosis* perhaps by as much a factor of two. Following the development of procedures for detection of the organism in stool samples, *Campylobacter jejune* has become recognized as a leading cause of acute bacterial gastroenteritis in humans. Raw milk is the food most commonly identified as the vehicle for foodborne outbreaks of *Campylobacterenteritis*.

Simple food poisoning is not the only problem caused by *Salmonella* and *Campylobacter*. Of hospitalized cases of *Salmonellosis*, up to 25% may be invasive, entering the blood stream and infecting other organ systems. The very young, the elderly, and immunocomprised people are especially suspectable to such septicemias, which sometimes have fatal consequences. Both *Salmonella* and *Campylobacter* can

also lead to a form of arthritis known as reactive arthritis, in about 1-3% of the cases, and can debilitate a person for six months or more.

Within the last ten years, microbiologists considered *Listeria* an obscure veterinary pathogen. *Listeria monocytogenes* has become a major concern in the food industry. Refrigeration has been an adequate barrier to control most bacterial growth. However, *Listeria* is a psychrotroph which grows well at 2-4°C., where competition by other organisms for nutrients is suppressed. The FDA has estimated that there are 18,050 cases of *Listeriosis*, with 425 deaths in the United States annually. The most common form of *Listeriosis* is meningitis. The mortality rate may reach 30% in the newborn, elderly, and people with defective immune systems. Pregnant women with meningitis often abort, and the infant, if living, usually develops a fatal septicemia. The clinical course develops and progresses suddenly, and the fatality rate may be as high as 70%. Although *Listeria* isolation, identification and enumeration methods have improved over the years since its discovery in the 1920s, these methods are still labor intensive and time consuming. The shortest methods take two weeks or more for confirmation of identity.

Hemorrhagic Escherichia coli is an organism of particular significance because there is an allowable level of the harmless types of *E. coli* in certain types of foods. In 1982, the Center for Disease Control identified *hemorrhagic E. coli* as the pathogen of an outbreak from hamburgers consumed in a fastfood restaurant chain, and since 1982, this *E. coli* strain has caused disease outbreaks in nursing homes, schools and other institutions. A test that detects all *E. coli* and cannot differentiate the *hemorrhagic* strain is almost useless in preventing foodborne infection by this organism.

Food processors and independent reference laboratories testing food for bacterial contamination mainly use traditional testing methods. Traditionally, identification of bacteria, particularly pathogens, is dependant upon methods that create artificial conditions which promote bacterial growth. The time for a bacterial cell to divide and its population to thereby double (the generation time) for most bacteria is one half-to-three hours; others require over 24 hours per generation. Thus, if one cell divides to form two cells every one half hour, after 12 hours, 2^{24} or 16 million cells could have been formed. To grow cells, various types of specific media is inoculated with the sample. Plates of the media are incubated and inspected for suspicious colonies. If a colony is present, small amounts of the colonies are added to different specialized biochemical media and permitted to incubate. In these media, the waste products of the bacteria's growth react with the media's chemicals and create visible color changes. Based upon observations of these media, highly skilled

microbiologists arrive at conclusions concerning the identification of the microorganism.

These traditional testing methods in the food, feed and cosmetic industries have been standardized by the Federal Food and Drug Administration (FDA) and the United States Department of Agriculture. The FDA publishes the Bacteriological Analytical Manual containing all of the methods now employed by the FDA laboratories to analyze the microbiological quality of food. These have come to be known as the "BAM" methods. Because these are the FDA's official protocols, most food processors and independent testing food laboratories use them to monitor food samples. The United States Department of Agriculture has also compiled a set of standardized tests that are similar to the BAM methods. Only slight differences are found between the testing recommendations of these agencies. The American Public Health Association works with the diary industry and publishes the Standard Methods for the Examination of Diary Products. This is a reference providing basic, traditional microbiological and chemical procedures for the analysis of milk, cheese, frozen and other diary products.

The traditional BAM methods require highly skilled microbiologists and have been shown to require the tedious and laborious task of determination of optimal methodology for each individual food type, with problems arising from the lack correlation between pure culture results and those results obtained from food products, feed and cosmetics. See, e.g., Fagerburg, D.J. and Evens, J.S., "Enrichment and Plating Methodology for Salmonella Detection in Food – a Review." J. Milk Food Technology 39: 628-646, 1976.

In a food there may be only a few pathogenic bacteria which, however, can grow into dangerous quantities on the food's nutrients during or after food processing and before consumption. If present in the food material, they may cause contamination of consumer or foodservice food preparation areas. Random sampling techniques are employed to sample large lots of processed food or food raw materials. These samples are blended in an appropriate growth medium , and liquified samples are then used to test for bacteria type following the BAM, USDA or other standardized protocol. The BAM and USDA methods will not provide even a screening result for three-or-four days from the day that samples are taken. Many food testing laboratories, which typically operate on a Monday-to-Friday basis, do not take samples after Wednesday, since test results would not be available on Friday. In those instances, only food products produced and sampled in the first three days of the week are screened for bacteria.

In the last 10-20 years, food processors have become increasingly efficient at producing larger and larger quantities of food from food raw materials in ever

widening varieties of food products. These products often have sensitive shelf life, particularly in the case of ready-to-eat meats and dairy products. Rapid and efficient methods of distribution have come into place to deliver, particularly shelf life sensitive products, to points of sale as quickly as possible to preserve most of the product's shelf life. This rapidity with which vast quantities of food products are produced and distributed to the consuming public has outpaced the technology for testing specimens of the food product for bacterial contamination by pathogens. During the three-to-four days needed for a test screening of a food product for bacterial contamination, the food usually has already left the plant and moved through the food distribution system to points of sale, particularly when the product is shelf-life sensitive. By the time test results for a pathogen are available, the food may already have been consumed. If test results are positive and necessitate a recall, the recall of the product must then also be directed to the consumer. This may be too late for the prevention of outbreak of a bacterial infection brought on by the pathogen. Even if the product is warehoused pending receipt of microbiological testing results before release for distribution, costs of warehousing for the lengths of time to get screening test results is a substantial cost of food processing which pressures profit margins and increases costs to consumers.

In recent years, originating with development of the absolutely safe foods necessary for astronauts in space flight required by the National Aeronautics and Space Administration, the Hazard Analysis and Critical Control Point (HACCP) system for preventing bacterial contamination of food during production has received a great deal of attention. HACCP involves a detailed analysis of every step in a process used to produce a product -- from arrival of a raw food or the live animal to a processing or slaughter plant, through final packaging and shipment. Each step is analyzed for potential hazards of contamination. Critical points in production most likely to cause food safety problems are identified, and these points are re-engineered and/or closely controlled in a rigid, formal manner. However, the slowness which current bacterial testing technology takes to give results has been an impediment to widespread adoption of HACCP systems in the American food processing system, much less in the rest of the world.

Accordingly, both for the benefit of the food consuming public and the food processor, there has been a great need for sensitive, specific microbiological tests which can be rapidly conducted and yield reliable bacterial screening results rapidly on food production and product samples.

Efforts to address this problem have largely centered upon attempts to adapt techniques developed for medical clinical laboratory microbiological testing. Two types of technology adapted from medical diagnostics technology have been applied to

microbiological testing of food. One is a DNA probe technology. This test requires several complex steps to produce a result. First, selectively enriched broth cultures pass through filters selectively trapping bacteria on their surfaces. Second, a chemical solution dissolves the outer layer of trapped bacteria , releasing cellular DNA into separate uncoiled strands. Third, the DNA strands are chemically fixed to the filter and exposed to a radioactive probe DNA. If the probe locates a complimentary section on the bacterial DNA, it will link and form a hybrid molecule. This hybrid now carrying a radioactive signal, can be measured by scintillation counter that can detect the beta emissions from the radioactive probe DNA. The DNA probe technology is also now offered in a colorimetric assay format where the hybrid forms a color instead of carrying a radioactive signal. The time required for a negative result is three days. The test is sensitive to detect a minimum bacterial organism population typically of one million organisms, or more.

Another system uses enzyme immunoassay (EIA) technology. Monoclonal antibodies are bound to the surface of beads made from a plastic coated ferrous metal. If the target bacterial antigens are present, they will bind to the antibodies on the surface of the beads. Washing the beads removes the unbound materials. The beads are then placed in a solution containing peroxidase-conjugated anti-bacterial antibodies. The conjugate binds to the bacterial antigens captured by the antibodies on the surface of the beads. A second washing step removes the unbound conjugate. The beads are then placed in a substrate solution. Appearance of color in the solution suggests the presence of target bacteria in the sample. The time to a negative result is three days. The test is sensitive to discern a minimal bacterial population of 100,000 organisms or greater.

These tests have not received widespread adoption among food processors because the time saving factor is but one day better than the traditional methodology in which investment already is placed, and because both require additional investment in sophisticated equipment and operation by highly skilled technologists. Both are suitable only for use in a testing laboratory.

Our invention departs from these prior technologies for testing foodstuff for bacteria and makes use of bacteriophage. In the past, bacteriophage have been employed in various assays, but usually only to determine the presence of antigen or an antibody in a serum or other bodily fluid sample.

In U.S. Patent 3,717,705 to *Haimovich, et al.*, the detection of antigens by the antibody inactivation of a bacteriophage conjugated with the antigen was described. An antigen was covalently bonded to a bacteriophage capable of growth on *E. coli*. The bacteriophage was inactivated by treating it with the antibody for the antigen. A

bacterium, which is a host for the bacteriophage, was inoculated with the inactivated bacteriophage, a culture of such bacterium was grown, and the amount of plaque formation in the culture was determined, with the degree of plaque formation being inversely proportional to the concentration of the antigen mixed with the antibody conjugate.

U.S. Patent 4,797,363 to *Teodorescu, et al.* described the use of bacteriophage as a carrier for antibodies. According to *Teodorescu*, the antibodies could be affixed to the bacteriophage by biotin-avidin recognition, coating the bacteriophage with avidin or biotin and binding to the antibody that has biotin or avidin, respectively. The bacteriophage could also be tagged with a labeling agent to assist in visualizing reaction of antigen to the antibodies on the bacteriophage.

We are aware of only one instance where bacteriophage have been employed in a test for bacteria. *Hubbard, et al.*, in International Application WO88/04326 published June 16, 1988, under the Patent Cooperation Treaty, discloses a method for identifying specific bacterial organisms by selecting a bacteriophage for a specific organism, affixing a labeling agent to the bacteriophage, contacting the labeled bacteriophage with a specimen of unknown organisms, binding the bacteriophage to the specific organism if present in the specimen, separating the bound bacteriophage and bacterium from the specimen, and testing the bacterium and bound bacteriophage to determine the presence of the label.

Our invention also makes use of very small solids such as already commercially available latex particles. Such particles previously have been suggested for immunological testing. *David, et al.* in U.S. Patent 4,486,530, in describing a "sandwich" enzyme immunometric assay technique for determination of the presence of antigenic substances in fluids using monoclonal antibodies, propose that antibodies could be bound to particles of latex. *David* suggests that when a quantity of particles to which a first monoclonal antibody is bound is admixed with a quantity of particles to which a second monoclonal antibody is bound, a milky suspension would result, but if a sample containing antigen for which the antibodies are specific were introduced to the suspension, agglutination of the particles would occur to form detectible particle clumps.

It is an object of this invention to provide a method and kit generally useful in microbiological testing to screen for the presence of specific bacteria types.

It is an object of this invention to provide a method and kit for screening food, feed, and cosmetic stuffs, in particular, for specific bacteria types. However, our invention is not limited to testing food, feed or cosmetic stuffs, but has application to

medical, environmental and veterinarian bacterial screening as well, and that is one of our objects.

It is an object of this invention to provide a method and kit for rapidly screening samples for the presence of organisms of a specific bacterial type, to within as little as a matter of minutes, depending upon the initial bacterial concentration in the sample.

It is an object of this invention to provide a method and kit for the screening of specific bacterial types which is so sensitive as to detect as few as ten's of the target organisms in a sample source. By comparison, the previously described EIA technology, the next quickest, commercially available test has a minimum detection sensitivity of roughly 100,000 organisms and takes three days.

It is an object of this invention to provide a method and kit for screening test samples for the presence of specific bacteria type which may be used reliably without specialized equipment and by quality control personnel under in-plant conditions.

It is an object of this invention to provide a method and kit which uses reagents that have a stable shelf life.

It is an object of this invention to provide a method and kit for the screening of a test sample for the presence of organisms of a specific bacterial type using a positive test indicator with a high "amplification" test signal for visual reading without instrumentation, but also adaptable to testing means using instrumentation, for example, colorimetric, enzymatic, fluorometric, luminescent, and radiometric means.

It is an object of this invention to provide a simple, highly specific, very sensitive, rapid, reliable method and kit, using stable reagents, for screening food and other sample types, including clinical, environmental and veterinarian samples, for the presence of organisms of a specific bacteria type.

These and other objects and advantages of our invention will be apparent from the following summary and description in detail of our invention.

Summary of the Invention

Our invention provides a method and kit for the determination of the presence of organisms of a specific bacteria type in a sample. Our method includes contacting a sample to be tested for presence of such organisms with both (i) a bacteriophage capable of attaching to such organisms, the bacteriophage having affixed to them one of two members of a specific binding pair, and (ii) a water-insoluble, water- suspendible solid phase carrier particle having affixed to it the second member of the specific binding pair. If organisms of the specific bacteria type are present in the fluid sample, a water-insoluble complex of at least (1) the organism, (2) the bacteriophages, and (3) the carrier particles is formed. The bacteriophage attach to the bacteria for which the

phage are specific, and the carrier particle(s) bind to the phage. We use the term "at least" when describing the complex above, because, actually, numerous phage attach to a single bacterium organism and numerous carrier parties bind to the attached phage, resulting in a cluster of a number of carrier particles to a single organism. This complex, if present, is then separated from any particles and bacteriophage in the test mixture not complexed to the specific organism. Testing to detect the complex, if present, is then conducted. If the testing detects the complex, the presence of the specific organisms in the sample is indicated.

The complexing or clustering phenomenon just mentioned is an important part of our invention, because it is the foundation for the specificity, sensitivity, and speed of our test. In effect, it identifies and enlarges the presence of a single organism to allow it to be isolated and identified.

Either the bacteriophage or the carrier particle has a labeling agent affixed to it. Preferably, the labeling agent is affixed to the carrier particle. Testing for the complex involves testing for the labeling agent.

Particularly with instrument label detection methods to which our invention is adaptable, the labeling agent may be affixed to the bacteriophage. However, where the labeling agent is a large polymer, such as a typical enzyme, and particularly where large numbers of such polymers are affixed to the phage, the phage has poor shelf stability, apparently because a dense concentration of large polymers compromises the integrity of the phage head.

In our preferred testing system, label detection is visual and requires no instrumentation. Here the labeling agent is affixed to the carrier particle. A labeled carrier particle in our invention is very shelf stable.

A singularly important reason in our invention for affixation of the labeling agent to the carrier particle is because many more of the labeling agents can be affixed to the carrier particle than to the typically much smaller bacteriophage. This facility for loading large numbers of labeling agents to a single carrier particle greatly increases the "signal" strength detectable compared to the signal strength which can be realized by the much smaller number of labeling agents the much smaller bacteriophage can carry. Coupled with the clustering phenomenon by which many of the labeled particles bind to many phage attached to the specific bacteria organism, a highly multiplied or amplified "signal" results from the presence of just one of the specific bacteria organisms. This amplification of the already strong signal permitted by densely loaded carrier particles gives our invention the sensitivity for even visual detection of the presence of on the order of ten's of organisms, rather than the ten's of thousands required for machine detection by the EIA system described above, or the

millions required by the above-described gene-probe technology, or the billions and trillions required by traditional methods.

This sensitivity means the system gives screening results very rapidly. Because our invention through its signal amplification is so sensitive as to detect on the order of ten's of organisms, there is no need for the many logarithms of organism replication and growth required by the currently available methods, in order even to have sufficient number of organisms to test or to detect. Further, because the test can be conducted so quickly and simply, the time for the testing regimen is reduced to a minimum.

The unique ability of our test system to "enlarge" individual bacterium organisms permits quick and simple testing for labeling. No special equipment is required. The cluster forming capability gives rise to a comparatively large particle. In the instance of *Listeria monocytogenes*, an organism having about a one micron length in its largest dimension, a complex larger than three to five microns is formed using carrier particles of the same nominal size as the organism. This permits the organism-bacteriophage-carrier particle complex to be retained on a filtration medium which will pass bacterial organisms other than those for which the phage is specific, as well as unattached phage, carrier particles and other particulate matter which are not complexed to phage attached to organisms. Thus, for a visual color test all that is required is a container for combining the phage and carrier particles with the sample, a suitable filtration medium, preferably a buffered wash solution to wash the filtration medium clean of passable size particles, and a reagent containing a substrate solution which will develop the color in cooperation with the labeling agent preferably on the carrier particle.

Because the test is so simple to conduct, trained microbiologists are not needed to do it, and testing could be adapted for in-plant or even in-field use, lending itself both to use for testing raw, in process, and finished product foodstuffs.

Because the test gives quick results, our invention makes it more practical for food, feed and cosmetic processors, and especially, makes it more feasible for food, feed and cosmetic processors to incorporate the principles of the Hazard Analysis and Critical Control Point (HACCP) system into their production systems.

The complex formed by our invention depends upon attachment of a bacteriophage to the specific bacterial organism to be detected if present, and upon binding of the carrier particle to the attached bacteriophage. The specific binding pair suitably may be a biotin, and avidin pair, or may be an antigen and an antibody to that antigen or similar binding compound pairs. Preferably, the binding pair is biotin and avidin, and preferably the biotin is affixed to the bacteriophage, rather than to the

carrier particle, because the avidin member of the pair is a much larger polymer than the biotin, and carriage of the avidin polymer by the carrier particle gives the longer shelf life stability possible with our invention. The small biotin polymer does not appear to disrupt the integrity of the bacteriophage head, and, accordingly, biotinylated bacteriophage have long shelf lives.

The concentration of avidinylated carrier particles contacted with a sample being tested for a specific bacteria-type and containing the biotinylated bacteriophage is important. If the concentration of the avidinylated carrier particle is too great, the particles will tend to agglutinate with each other in the reagent and compromise the reagent. Even if less than sufficient to agglutinate in the avidinylated carrier particle reagent, the concentration still may be so excessive, upon combination with a solution containing biotinylated bacteriophage, as to result in inter-binding of multiple carrier particles and bacteriophage not attached to target bacteria. This agglutination could give sizes large enough not to pass through a filtration medium chosen for the system, which would give false positive test results. However, if the concentration is too low, there are insufficient particles to form the carrier particle clustering complexes of this invention. Accordingly, at least where the carrier particles are avidinylated and the bacteriophage are biotinylated, the carrier particles are provided in suspension in an aqueous medium in a concentration sufficient in the medium to permit formation of the formed complex (of the organism, the bacteriophage and the carrier particle) with the biotinylated bacteriophage, yet insufficient in concentration for agglutination of the particles in the suspension to an extent which prevents their separation from the formed complex (of the organism, the bacteriophage and the carrier particles).

Suitably, the solid carrier particle has a size approximating the size of the organism to be detected; however, the particle size in its largest dimension may vary from as little as 0.2 to as much as 10 times the size of the organism, depending upon the method of separation employed. Where the carrier particle are about one micron in diameter and are formed of polystyrene latex, the carrier particles suitably are in aqueous suspension at a concentration ranging from about 1×10^6 to about 1×10^7 particles per mL.

The biotinylated bacteriophage are in suspension in an aqueous medium, in a concentration of bacteriophage sufficient for a ratio of bacteriophage to organisms of the selected bacteria type effective to produce sufficient of the complex (of the organism, bacteriophage and particle) for detection. Suitably, the ratio is sufficient for the detection of bacteria present in a sample at a concentration in the range from as little as ten colony forming units (CFU) of organisms and up.

Thus, in accordance with this invention, a method is provided for determining the presence of organisms of a specific bacterial type present in fluid, which comprises (a) selecting a bacteriophage which attaches to organisms of the specific bacteria type, (b) affixing a first binding agent to the bacteriophage to obtain a combining bacteriophage, (c) selecting a water-insoluble. water- suspendible particle of approximate size to the specific organism, (d) affixing a second binding agent and a labeling agent to the inert particle to obtain a labeling combining particle, the second binding agent being operative to bind with the first binding agent, the labeling agent optionally being connected to the second binding agent. Then, (e) the combining bacteriophage is added to the fluid sample being tested for presence of the organisms of the selected bacteria type, whereby if the organism is present, the combining bacteriophage attaches to the organism, and (f) the labeled combining particle is added to the sample, either with or after the combining bacteriophage of step (e), whereby the labeled combining particle binds with the combining bacteriophage, resulting; if the organism of the specific bacteria is present, in a complex of at least the organism, the bacteriophage, and the particle, such complex being of a larger size than the labeled combining particle or the labeled combining particle combined with the bacteriophage unattached to the organism. This is then followed by (g) separating the complex, if present, from the fluid, from labeled combining particles, and from labeled combining particles combined with combining bacteriophage unattached to the organisms, and (h) testing for the separated complex, if present, to detect the label, thereby to determine if organisms of the specified type are in the sample.

The method of this invention is particularly adapted to screening for bacteria types selected from the group consisting of *Salmonella*-type, *Listeria*-type, *Campylobacter*-type, *Bacillus*-type, hemorrhagic *Escherichia*-type, and *Shigella*-type bacteria or any other microorganism that is susceptible to phage binding.

The method thus involves, in a particular, a determination for the presence in a fluid of organisms of a bacteria type selected from the group consisting of *Salmonella*-type, *Listeria*-type, *Campylobacter*-type, *Bacillus*-type, *hemorrhagic Escherichia*-type, and *Shigella*-type bacteria, or any other microorganism that is susceptible to phage binding which comprises first contacting a sample of the fluid with (a) a measured amount of an aqueous suspension of biotinylated bacteriophage capable of attaching to organisms of a selected bacteria type, and (b) a measured amount of an aqueous suspension of a water-insoluble, water-suspendible avidinylated solid phase carrier particle labeled with an enzymatic colorimetric indicator, the carrier particle having a size approximating one micron (μm) in its largest dimension, whereby, if organisms of the selected bacteria type are present in the fluid sample in the predetermined minimum

concentration, a test mixture is formed containing a complex of at least (1) the organism, (2) the bacteriophage, and (3) the carrier particles, such complex having a size of at least three to five microns in its largest dimension. The test mixture is then deposited onto a top surface of a filtration medium having a pore size approximating, but not larger than, the size of the complex, for example, a pore size of about three to less than five microns for a complex not smaller than five microns. The filtration medium is then washed with a buffered aqueous medium, whereby particulate matters smaller than three to five microns in largest dimension are flushed through the filtration medium, and the complex, if formed, is retained on the medium. A color developing reagent, including a substrate for the enzymatic colorimetric indicator label, is then deposited onto the filtration medium surface. An indicator color is formed on the medium if the complex is present, thereby indicating the presence of organisms of the selected bacteria type in the sample.

A diagnostic kit for the determination of presence of organisms of a selected bacteria type fluid in accordance with this invention comprises (a) a first reagent including a bacteriophage capable of attaching to organisms of the selected bacteria type, a first member of a specific binding pair, preferably, biotin, being coupled to the bacteriophage, and (b) a second reagent including a second member of the specific binding pair, preferably avidin, coupled to a water-insoluble, water- suspendible solid phase carrier particle having a size approximating the size of such organisms. A labeling agent is affixed to at least to the bacteriophage in the first reagent or to the carrier particles in the second reagent, preferably to the carrier particles in the second reagent.

The diagnostic kit may further comprise a filter medium which retains on it a complex of at least the organism, the bacteriophage and the solid phase, the complex being retained on the filter medium, if such organisms are present in the fluid tested, by contacting the fluid with the first and second reagents and depositing the resulting mixture onto the medium. The diagnostic kit may further include a third reagent which tests for the presence of the labeling agent.

Description of the Drawings

Figures 1-4 are schematic or symbolic representations of the operation of this invention in successive stages or steps.

Figure 1 depicts a liquid sample in which labelled bacteriophage capable of attaching to the target organism under test are added to a liquid sample being tested for the presence of such organisms.

Figure 2 depicts the sample of Figure 1 after a water-insoluble, water-suspendible solid phase carrier particle is added to the liquid sample test.

Figure 3 schematically depicts the operation in which the water-insoluble complex of the target organism , the labelled bacteriophage and the carrier particle are separated from other particles, bacteria and bacteriophage.

Figure 4 schematically depicts a color producing reaction for detecting the complex.

Figure 5 schematically depicts a bacteriophage bound to the cell wall of a bacteria and coupled to a latex particle by means of avidin-biotin coupling agents.

Description of the Preferred Embodiment

Referring to the depictive drawings, a particular embodiment is illustrated with the understanding that it is exemplary of the more general concepts of this invention. In Figure 1, the reference numeral **10** indicates a field of a sample being tested for the presence of cells **11** of organisms of a specific bacterial type. In the drawings, this bacterial type is a *Listeria* organism, and cell **11** is labeled accordingly. Reference numeral **12** refer to cells of non-*Listeria*-type bacteria, and, also, are accordingly labeled. In Figure 1 and in the other drawings, the bacterial cells have a typical one micron longitudinal dimension. Reference numerals **13** refer to bacteriophage which have been added to sample **10**. The bacteriophage **13** are capable of attaching to *Listeria* cell **11**, but not non-*Listeria* cells **12**. As depicted in Figure 1, some of the bacteriophage **13** have attached to *Listeria* cell **11**. Referring to Figure 5, bacteriophage **13** comprises a head **14**, a tail **15**, tail fibers **16**, and a tail plate **17** viewable in the illustration. Tailplate **17** is shown attached to the cell wall of bacterial cell receptor site **24**. Bacteriophage **13** has affixed to its head **14**, one of two members of a specific binding pair, in this embodiment, biotin, represented schematically and indicated by reference numeral **18**. Bacteriophage **13** is illustrated in the drawings as having an approximate longitudinal dimension of 0.2 microns relative to the dimension of the bacterial cells.

In Figure 5, there also is depicted a water-insoluble, water- suspendible solid phase carrier particle **19**, suitably a latex sphere having a particle dimension approximating the longitudinal dimension of bacterial cell **11**, in these drawings, a diameter of about one micron. Figure 5 also schematically illustrates, coupled to latex sphere **19**, the second member of the specific binding pair complementary to member **18**. The second member, in this embodiment, avidin, is identified by reference numeral **20** as the schematic member **20**. The avidin member **20** is illustrated coupled

with a horseradish peroxidase (HRP) enzyme identified by reference numeral 21. The avidin/enzyme conjugate 20,21 is illustrated attached to latex sphere 19.

The specific binding pair 18,20 and the conjugate 20,21 are not to any scale with reference to bacteriophage 13 or latex sphere 19. Only a single binding pair is illustrated but in practice the bacteriophage 13 is covered with biotin molecules and sphere 19 is covered with avidin/HRP conjugate.

In accordance with the invention, bacteriophage 13 and water-insoluble, water-suspendible solid phase carrier particles 19 are contacted with the samples 10. Figure 2 depicts a field of sample 10 in which bacteriophage 13 and carrier particles 19 have been contacted with the sample to form a test mixture, which includes miscellaneous food particles or the like 22, non-*Listeria* cells 12, unattached bacteriophage 13, and solid carrier spheres 19 containing an avidin binding agent to which some bacteriophage 13 with a complimentary binding agent biotin have become attached, depicted generally by reference numeral 23. Also depicted in the field is a water-insoluble complex 25 comprising at least the organism of interest 11, bacteriophage 13, and solid carrier particles 19. A plurality of bacteriophage 13 are attached by base plate 17 to the cell wall receptor site of organism 11. As mentioned, avidin/enzyme conjugates cover the surface of solid carrier particles 19. As illustrated in the case of one of them in Figure 5, these avidin/enzyme conjugates couple through the avidin member to the biotin binding pair member on the head of bacteriophage 13. There are many of these biotin particles attached to the head of bacteriophage 14, and within stearic hinderance constraints, a plurality of the spheres 19 can bind with a single bacteriophage attached to cell 11. The spheres bound to the phage attached to the target organism represent the primary layer of bound spheres. The complex continues to enlarge as a result of chain like reaction of phage and spheres binding to the outer surfaces of the primary layer and thereby creating multiple outer layers. This produces a grape-like cluster of spheres about a single organism. With each of the spheres having a diameter of approximate dimension to that of the organism, a very large complex of a single bacteria, multiple bacteriophage, and multiple spheres is produced. This complex or cluster has an approximate diameter which is substantially larger than the size of the organism under test. Referring to Figure 3, a filtration medium indicated generally by reference numeral 27 is schematically shown in partial perspective section. Filtration medium 27 includes a filtration surface 26 defining a plurality of filtration pores 28 in contact with an underlying absorbent bed 29 comprised of a wicking matrix of fibers. In accordance with the invention, after the sample is contacted with the bacteriophage 13 and the solid phase carrier particles 19 to form complex 25, complex 25 is separated from any particles and bacteriophage not

complexed to organism 11. Suitably this is accomplished by pouring a liquid sample 10 onto surface 26 of filtration medium 27. The liquid is absorbed by the absorbent bed 29 through pores 28. Particles smaller than the diameter of pore 28 also pass through the pores into the absorbent bed where they are trapped. Suitably, a buffered washing solution is poured onto surface 26 after deposition of the sample 10 material containing the complex 25, to wash from surface 26 through pores 28 any remaining carrier particles 19, bacteriophage 13, non-specific bacterial cells 12, and other miscellaneous matter 22, leaving only the complex/cluster 25 atop the filtration medium surface.

Next, the surface of the filtration medium 26 is tested to detect whether any complex 25 is present on the surface, as will be the case if specific organism under test (organism 11 in the drawings) was present in sample 10. Referring to Figure 4, a substrate for the horseradish peroxidase affixed to the latex spheres has been poured onto the filtration surface. The horseradish peroxidase enzyme acts on the substrate and yields a colored byproduct that colors the spheres and, also, the filtration surface. This produces a visual indication of color and positively indicates that the organism under test, cell 11, was present in sample 10.

Bacteriophage specific to a particular bacterial species are prepared and biotinylated by well known methods. A particular bacteriophage is commercially available for the host bacteria. For example, *Listeria monocytogenes* is available as ATCC-23074 from the American Type Culture Collection, Rockville, Maryland, and the bacteriophage specific to such bacterial host is available from the same source as ATCC-23074B1. The following illustrates a procedure for preparation of a biotinylated bacteriophage reagent specific to *Listeria monocytogenes*, modified from Molecular Cloning (T. Maniatis, E.F. Fritsh and J. Sambrook; Cold Spring Harbor Laboratory, page 67 (1982)). The materials employed are: tryptic soy digest broth (TSB) prepared in deionized or distilled water; overnight broth cultures of the phage host in TSB (10 ml or greater); bacteriophage (approximately 1×10^8 plaque forming units (PFU)/mL) as crude lysate or as cleaned preparation; and 1.0 mM $MgSO_4$. A rotary shaker platform is used. An aliquot of the overnight *Listeria* broth culture is mixed with bacteriophage (crude lysate or purified phage) in a ration of 10 parts bacterial culture, 1 part bacteriophage suspension. This should yield an initial phage titer in this suspension of 1×10^7 PFU/mL. The suspension is mixed and incubated at 37°C. for 30 minutes to allow adsorption of the bacteriophage to the host organisms. The absorbed bacteria:bacteriophage suspension is diluted 1:40 into tryptic soy broth supplemented with 10 mM $MgSO_4$. The suspension is incubated at room temperature with rotary agitation (approximately 100 rpm) until the culture demonstrates clearing, indicating lysis. This can be a period from 6-18 hours. If the clearing phase is missed,

the unreleased bacteriophage can be liberated from the host cells by the addition of chloroform to yield 1% of volume. The chloroform treated suspension is incubated at room temperature for an additional 30 minutes with agitation to facilitate cell lysis. The bacteriophage are collected from the lysed suspension by filtration through a glass fiber prefilter to remove large bacterial debris followed by filtration through a 0.45 μm membrane filter to yield bacteria free phage in the supernatant. Cleared supernatants are stored at 4°C. in the crude state until further use or for long term storage. Long term storage of *Listeria* phage at 4°C. as crude lysate does not result in significant loss of titer for as long as one year. Greater titer losses occur after frozen storage. All crude bacteriophage preparations should be titered monthly.

To further purify the bacteriophage lysates by removing residual bacterial host contaminates, the following procedure may be employed, adapted from Manjatis. Molecular Cloning, *ibid*, p. 80. Materials employed are: crude phage lysate (titer should be at least 10^9 PFU/ml); sterile flasks or bottles; sterile oakridge tubes for 10,000 g centrifugation; NaCl solid; DN'ase and Rn'ase (1 mg/ml in deionized H_20); ice bath: and Polyethylene Glycol 6000 (PEG6000). Equipment used is a high speed centrifuge (Sorvall RC5B) with a SS34 angle head. Chill the crude bacteriophage lysate in an ice bath and add DN'ase and RN'ase to a final concentration of 1 μg/ml. This is mixed and incubated at 37°C. for 30 minutes to destroy residual free DNA and RNA in the lysate suspension. After incubation with DN'ase and Rn'ase, solid NaCl is added to a final concentration of 1M. This is swirled to dissolve, and incubated on ice for one hour. After incubation, the suspension is centrifuged at 10,000 g for 10 minutes to pellet the precipitated bacterial debris from the suspension. Do not run centrifuge with brake engaged. Immediately after the centrifuge stops, the supernatant is collected into a fresh bottle or flask. Do not allow centrifuged material to remain in the tubes for long periods since the pellet will dislodge and return to solution. To the collected supernatant, solid PEG6000 is added to a final concentration of 10% (w/v). This is dissolved and incubated on ice for at least one hour. After incubation, this is centrifuged at 10,000 g for 10 minutes. Run centrifuge without brake and remove tubes immediately after termination of run. Supernatant is poured off and residual fluid is removed by standing the centrifuge tubes inverted on absorbent material for at least five minutes. Precipitated bacteriophage is collected by gently washing the tubes with phosphate buffered saline to yield a final volume of 2% of the original lysate volume (e.g., original lysate - 1 liter; final lysate is collected in 20 ml of PBS). The precipitate is easily collected using a short pasteur pipet. Each tube should be washed two times. therefore, volumes should be calculated accordingly. The resuspended precipitate is finally extracted with chloroform. Extracts are centrifuged at 5000 g for five minutes

to separate the chloroform and aqueous phases. The centrifuge should be run without braking and all tubes should be removed immediately at termination of the run. The upper aqueous phase is collected with a short pasteur pipet, taking care not to disturb the dense white pellicle between the chloroform and aqueous phases. The cleaned phage may be stored in glass vials at 4°C. for indefinite periods and should be assayed on a monthly basis to ascertain phage titer.

Using a cleaned bacteriophage preparation, the bacteriophage are biotinylated, suitably as follows: (1) dilute phage to 10^9 - 10^{10} PFU/ml in Carbonate/bicarbonate buffer; (2) prepare a 1 mg/ml solution of NHS-biotin in 1 ml of Dimethylformamide (DMF). This preparation may be stored at 4°C. and reused; (3) mix phage with NHS-biotin solution to yield approximately 2 x 10^6 biotin molecules/phage. Example: 10 µl of 1 mg/ml NHS-biotin/ 5 ml of reaction mixture = approximately 3.4 x 10^{15} biotin molecules per ml; (4) Incubate at room temperature with gentle stirring for 1 hour; (5) dialyze versus three changes of 2 liters of PBS at 4°C. for approximately 36 hours; (6) collect dialysate, filter (0.45 µM) for sterility and titer; and (7) the biotinylated bacteriophage is stored at 4°C. until use.

A method of preparing a carrier particle reagent using polystyrene latex particles and conjugating the particles with an avidin - horseradish peroxidase conjugate follows:

1. To a 3 mL screw top vial with a rubber cap liner add the following ingredients in the order listed:
   a. 1.0 mL of 0.05 M, Phosphate buffer, pH 7.3;
   b. 100 µL of Strepavidin/Horseradish Peroxidase Conjugate (TAGO, Inc. Burlingame, California, Catalog No. 6467); and
   c. 75 µL of 1µm latex spheres (sulfate) (Interfacial Dynamics Corp., Portland, Oregon, Catalog No. 109-86-15-218).
2. Place the vial on a tube rocker and mix gently for 10 minutes at room temperature, approximately 25°C.
3. Decant the contents of the vial into a 100 mL beaker. Wash the vial two times with 1-2 mL of 0.05 M, Phosphate buffer, pH 7.3 and pour the contents into the beaker.
4. Dilute the contents of the beaker with an additional 35 to 40 mL of 0.05 M, Phosphate buffer, pH 7.3.
5. Evenly divide the diluted latex suspension into conical centrifuge tubes. (Corning Polystyrene No. 25300).
6. Centrifuge for 15 minutes with centrifuge at 2,500 - 3,000 RPM. (Clay-Adams Safeguard Centrifuge, Model 0049).

7. Aspirate the supernatant with a pasteur pipette and flexible tubing attached to a vacuum source. Add 4-6 mL of 0.05 M. Phosphate buffer, pH 7.3 to each tube and resuspend the latex.
8. Repeat steps 6 and 7 two more times.
9. After the final washing resuspend the latex by adding 1.0 mL of 0.05 M. Phosphate buffer, pH 7.3 to one of the centrifuge tubes and resuspend the latex. Transfer this suspension to the next tube and repeat until the latex from all the tubes has been resuspended in the 1.0 mL volume of buffer.
10. Store at room temperature.

Example 1

Stability of the Conjugated Phage And Enzyme Labeled Latex Reagents

The stability of conjugated phage and avidin/enzyme labeled latex reagents was determined according to the following methodology:

All reagents were stored in concentrated form at room temperature.

All reagents were diluted 6-24 hours prior to assay in the following manner:

Phage Reagent No. 2:28: Dilute 1:400 in 0.01M phosphate buffered saline (PBS), pH 7.4;

Latex Reagent: Dilute 1:1600 in PBS supplemented with 0.025% Tween 20;

Bacterial cultures were incubated overnight in Listeria Enrichment Broth (DIFCO Laboratories, Inc., Detroit, Michigan) and then were diluted to 10^8 CFU/ml (1 McFarland turbidity). This suspension was then diluted serially to 10^4 CFU/ml in Saline/10% broth diluent. The assay conditions used were as follows: (a) mix 100 ul of bacterial suspension (10^4 CFU/mL) with 100 μl of phage reagent, shake gently for 10 sec. and incubate at room temperature for three minutes; (b) add 100 μl of latex reagent, shake gently for 10 sec. and incubate at room temperature for two minutes; (c) pour mixture onto five micron (5μm) nylon membrane and wash with PBS/Tween wash buffer; and (d) add substrate solution and wait three to five minutes for color to

develop. Blue-green color is positive, and intensity of color determines plus (+) level. The results obtained follow in Table 1.

TABLE 1

Time	Latex Reagent Batch No.	Phage Reagent Batch No.	Reaction Intensity	Negative Control
0	1	2:28	+3	-
1	1	2:28	+3	-
2	1	2:28	+3	-
4	1	2:28	+3	-
8	1	2:28	+3	-
12	1	2:28	+3	-
16	1	2:28	+3	-
20	2	2:28	+3	-
24	2	2:28	+3	-
36	2	2:28	+3	-
72	2	2:28	+3	-
76	2	2:28	+3	-

These data illustrate the stability of both the biotinylated phage reagent and the avidin/enzyme labelled latex reagent. The same phage reagent was used throughout the course of the study. Latex reagent batch number 1 was used for the first six months of the study and latex reagent batch number 2 was used from week 20 through week 76.

Example 2

Determination of the Specificity of a *Listeria* Species Specific Biotinylated Bacteriophage Employing an Avidinylated Enzyme Labeled Latex Particle Reagent System

Following the same test methodology and reagents and described in Example 1, the specificity of a *Listeria* specific biotinylated bacteriophage reagent employing the avidinylated horseradish peroxidase enzyme labeled latex particle reagent system of this invention was tested. Testing results is interpreted as follows: Blue-green color formation is considered positive. absence of color formation is considered negative. The results obtained follow in Table 2.

TABLE 2

Organism	No. of Specimens	No. of Tests Done	Testing Results	
			Positive	Negative
L. monocytogenes 1a	2	8	8	0
L. monocytogenes 1b	2	8	8	0
L. monocytogenes 2	2	8	8	0
L. monocytogenes 3b	2	8	8	0
L. monocytogenes 4b	2	8	8	0
L. monocytogenes (untyped)	6	24	24	0
L. murrayi	4	8	8	0
L. grayi	4	8	8	0
L. seeligeri	4	8	8	0
L. welshimeri	4	8	8	0
L. ivanovii	4	8	8	0
L. innocua	4	8	8	0
Salmonella	11	22	0	22
Enterococcus	10	20	0	20
Streptococcus	8	16	0	16
Staphylococcus	8	16	0	16
Lactobacillus	5	10	0	10
Candida	5	10	0	10
Bacillus	6	12	0	12
Brochothrix	1	2	0	2

These data illustrate the absence of cross reactivity with any of the non-Listeria species in this study. A negative control was run with each test. The assay system correctly identified Listeria spp. and did not react with any of the non-Listeria spp.

Example 3
Determination of the Sensitivity of a
Biotinylated Bacteriophage and Enzyme Labeled
Avidinylated Latex Particle Assay System

All reagents were stored in concentrated form at room temperature and were diluted 6-24 hours prior to assay in the following manner:

Phage Reagent No. 2:28: Dilute 1:400 in 0.01M phosphate buffered saline (PBS), pH 7.4;

Latex Reagent: Dilute 1:1600 in PBS supplemented with 0.025% Tween 20;

Bacterial cultures were incubated overnight in Listeria Enrichment Broth (DIFCO Laboratories, Inc., Detroit, Michigan) and then were diluted to 10^8 CFU/ml (1 McFarland turbidity). This suspension was then diluted serially to 10^4 CFU/ml in Saline/10% broth diluent. The assay conditions used were as follows: (a) mix 100 ul of bacterial suspension (10^4 CFU/mL) with 100 μl of phage reagent, shake gently for 10 sec. and incubate at room temperature for three minutes; (b) add 100 μl of latex reagent, shake gently for 10 sec. and incubate at room temperature for two minutes; (c) pour mixture onto five micron (5μm) nylon membrane and wash with PBS/Tween wash buffer; and (d) add substrate solution and wait three to five minutes for color to develop. Blue-green color is positive, and intensity of color determines plus (+) level. The results obtained follow in Table 3.

TABLE 3

Months	Phage Reagent	Latex Batch No.	Test Suspensions (CFU/mL)								
			10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1	0
0	2:28	1	+2	+2	-3	+3	+3	+3	+2	+2	-
5	2:28	1	+2	+2	-3	+4	+4	+3	+3	+1	-
10	2:28	2	+2	+2	+3	+3	+3	+4	+3	+2	-
21	2:28	2	+2	+2	-3	+3	+3	+3	+2	+1	-

These data illustrate the minimum number of colony forming units (CFUs) detectable for zero CFUs.

Example 4
Determination of the Efficacy of Biotinylated
Bacteriophage and Enzyme Labeled Avidinylated Latex
Particle Reagent Assays System in Food Cultures

All reagents were stored in concentrated form at room temperature and were diluted 6-24 hours prior to assay in the following manner:

Phage Reagent No. 2:28: Dilute 1:400 in 0.01M phosphate buffered saline (PBS), pH 7.4;

Latex Reagent: Dilute 1:1600 in PBS supplemented with 0.025% Tween 20;

Bacterial cultures were incubated overnight in Listeria Enrichment Broth (DIFCO Laboratories, Inc., Detroit, Michigan) and then were diluted to 10^8 CFU/ml (1 McFarland turbidity). This suspension was then diluted serially to 10^4 CFU/ml in Saline/10% broth diluent.

For Assay: Ground Turkey was obtained from the local supermarket. Food quality control literature suggests that this food product contains significant quantities of the *Listeria* organism in its uncooked state.

25 grams of ground turkey was weighed and mixed into 225 ml of *Listeria* Enrichment Broth in a sterile plastic bag. The culture was manipulated by squeezing for five minutes to distribute the meat fiber material into the culture medium. Samples were taken as indicated and assayed for reactivity in the Listeria detection assay as compared to known positive control cultures. All turkey samples were diluted 1:4 in Saline/10% broth diluent prior to assay to facilitate filterability.

The following steps were used: (a) mix 100 μl of bacterial suspension with 100 μl of phage reagent, shake gently for 10 sec. and incubate at room temperature for three minutes; (b) add 100 μl of Latex reagent, shake gently for 10 sec. and incubate at room temperature for two minutes; (c) pour mixture onto five micron (5μm) nylon membrane and wash with PBS/Tween wash buffer; and (d) add substrate solution and wait three minutes for color to develop. Blue-green color is positive, intensity of color determines plus level. The results obtained follow in Table 4.

TABLE 4

Time in Culture

Week	1 Hour		6 Hours		20 -24 Hours		42 - 48 Hours	
	Sample	Control	Sample	Control	Sample	Control	Sample	Control
0	No Test	No Test	+3	+3	+3	+3	No Test	No Test
12	+2	+2	+3	+2	+3	+2	No Test	No Test
20	+2	+3	+2	+3	+3	+3	+3	+2

Positive Control = L. monocytogenes at 10^4 CFU/mL (100 µL per test)

No Test indicates that the sample was not tested at that time interval.

These data illustrate the assay's ability to detect the target organism within 1 hour of starting preenrichment of a sample that has not undergone any type of bacteriocidal treatment.

Having now described this invention and the manner of making and using it, applications and embodiments of it other than set forth herein but still within the spirit and scope of this invention as claimed or as equivalent to what is claimed will occur to those in the art. These are intended encompassed by the claims, which now follow.

WHAT IS CLAIMED IS:

1. A method for the determination of the presence of a specific bacteria in a sample, comprising:

(a) contacting a sample to be tested with

(i) bacteriophage capable of attaching to said specific bacteria, said bacteriophage having affixed thereto one of two members of a specific binding pair, and

(ii) water-insoluble, water-suspendible solid phase carrier particles having affixed thereto the second member of said specific binding pair,

to form a test mixture having water-insoluble complexes of at least (1) said specific bacteria, (2) said bacteriophage, and (3) said solid phase particles, if said specific bacteria are present in said fluid sample,

(b) separating said complexes from solid phase particles and bacteriophage in said test mixture not complexed to said specific bacteria, and

(c) testing to detect said complexes, thereby to determine if said specific bacteria are present.

2. A method for the determination of the presence of a specific bacteria in a sample, comprising:

(a) contacting a sample to be tested with

(i) bacteriophage capable of attaching to said specific bacteria, said bacteriophage having affixed thereto one of two members of a specific binding pair, and

(ii) water-insoluble, water-suspendible solid phase carrier particles having affixed thereto the second member of said specific binding pair,

a labeling agent being affixed to at least one of said bacteriophage or said carrier particle,

to form a test mixture having water-insoluble complexes of at least (1) said specific bacteria, (2) said bacteriophage, and (3) said solid phase particles, if said specific bacteria are present in said fluid sample.

(b) separating said complexes from solid phase particles and bacteriophage in said test mixture not complexed to said specific bacteria, and

(c) testing to detect said complexes. thereby to determine if said specific bacteria are present.

3. The method of claim 2 in which said specific binding pair are biotin and avidin.

4. The method of claim 2 in which said specific binding pair are an antigen and an antibody thereto.

5. The method of claim 2 in which said labeling agent is affixed to said carrier particle.

6. The method of claim 2 in which said particle has a size approximating the size of said specific bacteria.

7. The method of claim 2 in which said particle has a size of from about 0.2 to about 10 times the size of said specific bacteria.

8. The method of claim 2 in which said particle size is approximately 1 micron.

9. The method of claim 2 in which in step (b) said complexes are separated by filtration retaining said complexes on a filtration medium and passing particles not complexed to said specific bacteria through said medium.

10. A method for the determination of the presence of a specific bacteria in a fluid, comprising:

(a) contacting said fluid with

(i) a measured amount of biotinylated bacteriophage capable of attaching to said specific bacteria, and

(ii) a measured amount of avidinylated water-insoluble, water-suspendible. solid phase, labeled carrier particles in suspension in an aqueous medium,

to form water-insoluble complexes of at least (1) said specific bacteria. (2) said bacteriophage, and (3) said carrier particles, if said specific bacteria are present in said fluid, said aqueous suspension having a concentration of such particles sufficient to

permit formation of said complexes if said specific bacteria are present in said fluid, and insufficient for agglutination of said particles in said suspension to an extent preventing their separation from said complex in step (b)

(b) separating said complex from said fluid and any carrier particles not complexed with said specific bacteria, to obtain a segregate of said complex if said specific bacteria are present, and

(c) testing for said segregate to detect said label, thereby to determine if said specific bacteria are present.

11. The method of claim 10 in which said avidinylated labeled carrier particles are in suspension in an aqueous medium from which suspension said measured amount is taken. said aqueous suspension having a concentration of such particles (i) sufficient to permit formation of said complex in step (a) if said specific bacteria is present in said sample and (ii) insufficient for agglutination of said particles in said suspension to an extent preventing their separation from said complex after said step (a).

12. The method of claim 10 in which said avidinylated labeled carrier particles are in suspension in an aqueous medium from which said measured amount is taken. said aqueous suspension having a concentration of such particles ranging from about 1×10^6 to about 1×10^7 particles per mL.

13. The method of claim 10 in which said avidinylated labeled carrier particle has a size in its largest dimension approximating the size of said specific bacteria.

14. The method of claim 10 in which said particles are polystyrene or other polymer/copolymer spheres having a particle size of about one micrometer.

15. The method of claim 10 in which said label is a member selected from the group consisting of a radioactive isotope, an enzyme, a luminescent, a chromogenic and a fluorogenic material, and said step (c) is by means selected from the group consisting of radiometric, enzymatic, luminescence, colorimetric and fluorometric means.

16. The method of claim 15 in which said label is an enzymatic colorimetric indicator.

17. The method of claim 16 in which said label is horseradish peroxidase.

18. The method of claim 17 in which the method of testing said segregate to detect horseradish peroxidase includes contacting said segregate with a peroxidase chromogenic substrate.

19. The method of claim 15 in which said label is a fluorescent chromophore.

20. The method of claim 19 in which said label is fluorescein.

21. The method of claim 15 in which said label is the radioactive isotope ^{125}I.

22. The method of claim 10 in which the biotinylated bacteriophage are suspended in an aqueous medium from which said measured amount is taken, said bacteriophage suspension having a concentration of bacteriophage sufficient for a ratio of bacteriophage to specific bacteria, if present, effective to produce sufficient of said complexes for detection of said label in step (c).

23. The method of claim 22 in which said ratio is sufficient for detection of bacteria present in said sample at a concentration in the range from as little as 10 CFU.

24. The method of claim 10 in which said bacteriophage is species specific.

25. A method of claim 10 in which the bacteriophage is genus specific.

26. The method of claim 10 in which the bacteriophage is specific for bacteria selected from the group consisting of *Salmonella*, *Listeria*, *Campylobacter*, *Bacillus*, hemorrhagic *Escherichia*, and *Shigella* bacteria or any microorganism that is susceptible to bacteriophage binding.

27. The method of claim 10 in which said complex is separated by filtration retaining said complex on a filtration medium.

28. The method of claim 27 in which said complex is larger than 3 - 5 μm.

29. The method of claim 28 in which said filtration medium has a pore size of about 3 - 5 μm.

30. A method for the determination of the presence in a fluid of specific bacteria, comprising:

(a) contacting a sample of said fluid with

(i) a measured amount of an aqueous suspension of biotinylated bacteriophage capable of attaching to specific bacteria, and

(ii) a measured amount of an aqueous suspension of a water-insoluble, water-suspendible avidinylated solid phase carrier particle labeled with an enzymatic colorimetric indicator, said carrier particle having a size approximating the size of said specific bacteria, thereby, if said specific bacteria are present in said fluid sample in a predetermined minimum concentration, to form a test mixture containing complexes of at least (1) said specific bacteria, (2) said bacteriophage, and (3) said solid phase carrier particles, said complex having a size of at least about 3 - 5 times, said aqueous suspension of avidinylated particles having a concentration of such particles sufficient to permit formation of said complexes if said specific bacteria are present in said sample, and insufficient for agglutination of said particles in said suspension to an extent preventing their separation from said complex in step (c)

(b) depositing said test mixture onto a filtration medium having a pore size of about 3 - 5 times the size of said bacteria,

(c) washing said filtration medium after step (b) with an aqueous medium, whereby particulate matter smaller than 3 - 5 times the size of said bacteria is flushed through said filtration medium and said complexes are retained on said filtration medium if said complexes were formed in step (a), and

(d) depositing a color developing reagent including substrate for said enzymatic colorimetric indicator onto said filtration medium, whereby an indicator color is formed on said medium if said complexes are present, thereby indicating the presence of specific bacteria in said sample.

31. A method of determining the presence of specific bacteria present in a fluid, which comprises:

(a) selecting a bacteriophage which attaches to a specific bacteria,

(b) affixing a first binding agent to said bacteriophage to obtain a combining bacteriophage,

(c) selecting a water-insoluble, water-suspendible solid phase particle of approximate size to said specific bacteria,

(d) affixing a second binding agent to said solid phase particle to obtain a combining particle, said second binding agent being operative to bind with said first binding agent,

(e) affixing a labeling agent to said solid phase particle or to said second binding agent to obtain a labeled combining particle,

(f) contacting said combining bacteriophage with a fluid to be tested for presence of said specific bacteria, whereby if said specific bacteria are present said combining bacteriophage attach to said specific bacteria,

(f) contacting said labeled combining particle with said fluid to be tested, either with or after said combining bacteriophage in step (e), whereby said labeled combining particles bind with said combining bacteriophage, resulting, if said specific bacteria is present, in complexes of at least (1) said specific bacteria, (2) said bacteriophage, and (3) said solid phase particles, said complexes being of a larger size than said labeled combining particle or said labeled combining particle combined with combining bacteriophage unattached to said specific bacteria,

(g) separating said complexes from said fluid and from labeled combining particles and labeled combining particles combined with combining bacteriophage unattached to a said specific bacteria, and

(h) testing for said complexes, if present, to detect said label, thereby to determine if said specific bacteria are present.

32. The method of claim 31 in which said label is a member selected from the group consisting of a radioactive isotope, an enzyme, a luminescent, a chromogenic and a fluorogenic material, and said step (c) is by means selected from the group consisting of radiometric, enzymatic, luminescence, a colorimetric and fluorometric means.

33. The method of claim 31 in which the bacteriophage is specific for bacteria selected from the group consisting of *Salmonella*, *Listeria*, *Campylobacter*, *Bacillus*, hemorrhagic *Escherichia*, and *Shigella* bacteria.

34. The method of claim 31 in which said first binding agent is biotin and said second binding agent is avidin.

35. A diagnostic kit for the determination of the presence of specific bacteria in a fluid, comprising:

(a) a first reagent including a bacteriophage capable of attaching to specific bacteria, a first member of a specific binding pair being coupled to said bacteriophage,

(b) a second reagent including a second member of said specific binding pair coupled to a water-insoluble, water- suspendible solid phase carrier particle having a size approximating the size of said specific bacteria, a labeling agent being affixed at least to said bacteriophage in said first reagent or to said carrier particle in said second reagent.

36. The diagnostic kit of claim 35, further comprising a filter medium which retains thereon complexes of at least (i) said specific bacteria, (ii) said bacteriophage, and (iii) said solid phase, said complexes being formed and retained on said filter medium, if said specific bacteria are present in said fluid, by contacting said fluid with said first and second reagents and depositing the resulting mixture onto said filter medium.

37. The diagnostic kit of claim 35 further comprising a third reagent which tests for the presence of said label.

38. The diagnostic kit of claim 36 further comprising a third reagent which tests for the presence of said label, for deposit onto filter medium after said mixture is poured onto the said filter medium.

39. The diagnostic kit of claim 35 in which said first member of a specific binding pair is biotin and said second member of such specific binding pair is avidin, and in which said particles are in suspension in an aqueous medium, such aqueous suspension having a concentration of such particles (i) sufficient to permit formation of said complexes if the specific bacteria are present in said fluid, and (ii) insufficient for agglutination of said particles in said suspension to an extent preventing their separation from said complexes.

40. The diagnostic kit of claim 35 in which said particles are suspended in an aqueous medium, such aqueous suspension having a concentration of said particles ranging from about 1×10^6 to about 1×10^7 particles per mL.

41. The diagnostic kit of claim 35 in which said bacteriophage are suspended in an aqueous medium, said bacteriophage suspension having a concentration of bacteriophage sufficient for a ratio of bacteriophage to specific bacteria, if present in a test sample, effective to produce sufficient of said complex for detection of said label.

42. The diagnostic kit of claim 41 in which said ratio is sufficient for detection of bacteria present in said sample at a concentration in the range from as little as 10 CFU.

43. The diagnostic kit of claim 35 in which said first member of a specific binding pair is biotin and said second member of such specific binding pair is avidin.

44. The diagnostic kit of claim 35 in which said labeling agent is a member selected from the group consisting of a radioactive isotope, an enzyme, a luminescent, a chromogenic and a fluorogenic material.

45. The diagnostic kit of claim 35 in which said labeling agent is affixed to said particle.

46. The diagnostic kit of claim 37 in which said labeling agent is an enzymatic colorimetric indicator and said third reagent includes a substrate for said enzymatic colorimetric indicator.

47. The diagnostic kit of claim 46 in which said indicator is horseradish peroxidase and said substrate is a peroxidase chromogenic substrate.

48. The diagnostic kit of claim 35 in which said bacteria-type is selected from the group consisting of *Salmonella*, *Listeria*, *Campylobacter*, *Bacillus*, hemorrhagic *Escherichia*, and *Shigella* bacteria.

49. The diagnostic kit of claim 36 in which said filter medium has a pore size of about 3 - 5 microns .

50. A diagnostic kit for the determination of the presence of specific bacteria in a fluid sample, said bacteria being selected from the group consisting of *Salmonella*, *Listeria*, *Campylobacter*, *Bacillus*, hemorrhagic *Escherichia*, and *Shigella* bacteria, comprising:

(a) a first reagent including a biotinylated bacteriophage capable of attaching to said selected bacteria, and

(b) a second reagent including water-insoluble, water-suspendible avidinylated solid phase particles in aqueous suspension labeled with an enzymatic colorimetric indicator and having a size approximating the size of said specific bacteria, said aqueous suspension having a concentration of such particles sufficient to permit formation of said complex of (1) the specific bacteria, (2) the bacteriophage, and (3) the solid phase particles if said specific bacteria are present in said sample, and insufficient for agglutination of said particles in said suspension to an extent preventing their separation from said complex.

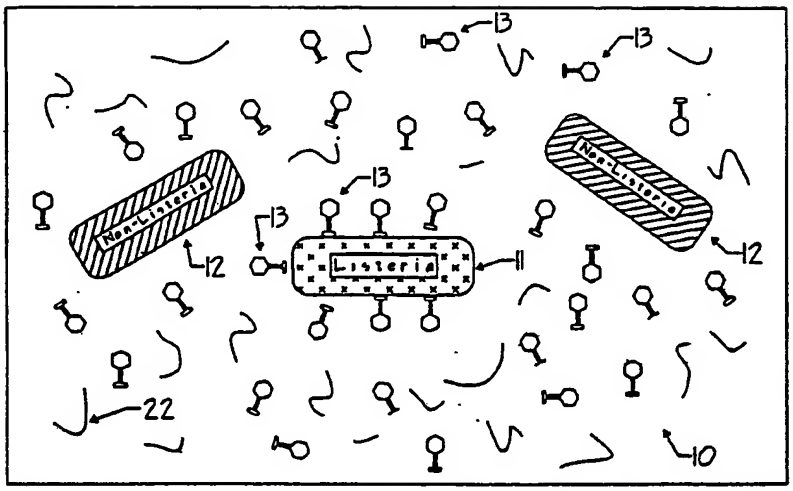
51. The diagnostic kit of claim 50 further comprising a filter medium having a pore diameter of about 3 - 5 microns.

52. The diagnostic kit of claim 50 further comprising a third reagent which includes a substrate for said enzymatic colorimetric indicator.

53. The method of claim 1 in which said first member of a binding pair is biotin and said second member is avidin.

54. The method of claim 2 in which said first member of a binding pair is biotin and said second member is avidin.

Fig. 1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13
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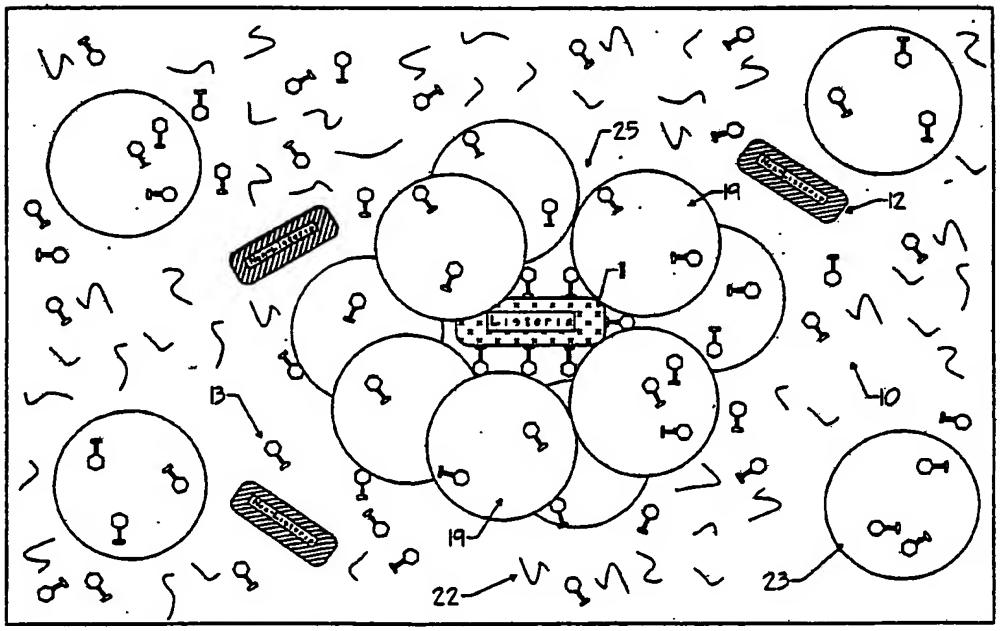
Non-Listeria
Listeria
12
12
11
22
10

Fig. 2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Non-Listeria
Listeria
1
13
10
19
22
23

Fig. 3

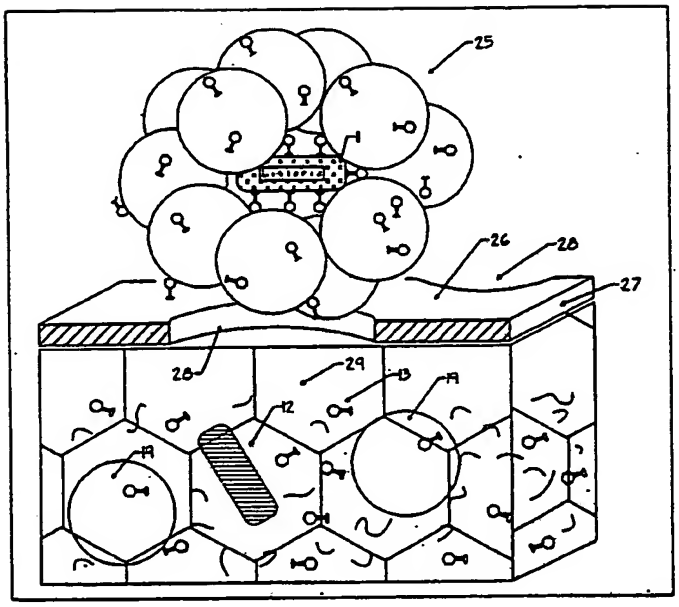


Fig. 4

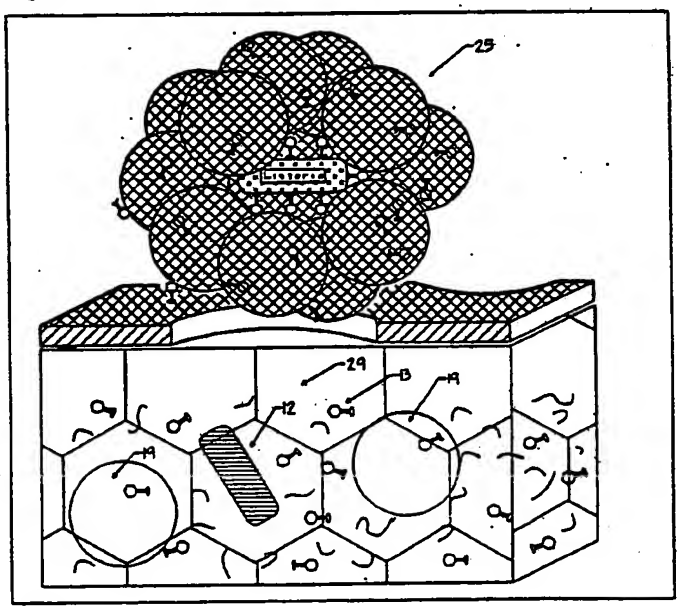
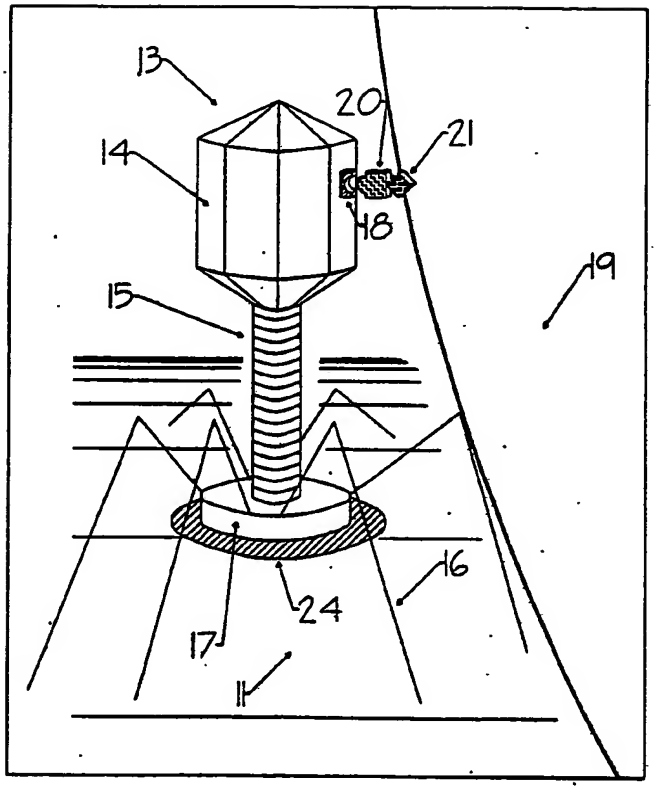


Fig. 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01627

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/70; G01N 33/569
US CL :435/5, 7.32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.32, 7.35, 7.37, 7.5, 7.9, 975; 436/518, 531, 534

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG; search terms: bacteriophage?, (avidin? or biotin?), (particle# or bead# or microparticle# or microsphere#)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,772,550 (Greenquist) 20 September 1988, see entire document, especially see column 7, line 11 to column 8, line 22 and column 9, lines 9-23	4, 6-8, 13-14, 16-18, 21, 28-52
Y	US, A, 4,797,363 (Teodorescu et al) 10 January 1989, see entire document, especially see Abstract, column 3, lines 43-51 and column 5, lines 33-49).	1-54
Y	WO 88/04326 (Hubbard et al) 16 June 1988, see entire document, especially see page 2, line 1 to page 3, line 3, page 5, first full paragraph and claims 3, 6, 9 and 10.	1-54

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 May 1993	Date of mailing of the international search report 02 JUN 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CAROL E. BIDWELL Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01627

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochimica et Biophysica Acta, Volume 728, issued 1983, Kaplan et al, "The Selective Detection of Cell Surface Determinants by Means of Antibodies and Acetylated Avidin Attached to Highly Fluorescent Polymer Microspheres", pages 112-120, especially see page 113, first column, first full paragraph.	1-54